DATA EVALUATION RECORD

2,4-DB

Study Type: OCSPP No Guideline; OECD 412; 28-Day Inhalation Toxicity Study in the Rat

EPA Contract No. EP-W-16-018
Task Assignment No. 32-2-004 (MRID 50624901)

Prepared for
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This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CDM/CSS-Dynamac Joint Venture personnel. Contractor's role did not include establishing Agency policy.

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DATA EVALUATION RECORD

STUDY TYPE: Subchronic (28-Day) Inhalation Toxicity in Rats; OPPTS 870.3465; OECD 412.

PC CODE: 030801 **DP BARCODE:** D448044

TXR#: 0057811

TEST MATERIAL (PURITY): 2,4-DB (97.74% a.i.)

SYNONYMS: 4-(2,4-dichlorophenoxy)butanoic acid

CITATION: Hoffman, G.M. (2018) 2,4-DB technical acid: a 4-week inhalation toxicity study in rats with an up to 4-week recovery period. Envigo CRS, Inc., East Millstone, NJ. Laboratory Study Number: FC01PC, July 3, 2018. MRID 50624901. Unpublished.

SPONSOR: 2,4-DB Task Force, represented by: Data Management Group, Inc., 101 Northway Court, Raleigh, NC.

EXECUTIVE SUMMARY: In a 28-day, nose-only, inhalation toxicity study (MRID 50624901), groups of 28 male Hsd:Sprague Dawley rats/concentration were exposed to 2,4-DB (97.74% a.i.; Lot # 80006961) by nose-only inhalation at concentrations of 0, 0.02, 0.05, or 0.20 mg/L for six h/day, five days/week for a total of 20 days. Ten animals/concentration were assigned to the terminal necropsy groups, and six animals/concentration/week were assigned to the recovery necropsy groups after Weeks 1, 2, and 4 of recovery.

There were no effects of treatment on mortality, clinical signs, body weight/body weight gain, food consumption, ophthalmoscopic examinations, hematology, clinical chemistry, urinalysis, organ weights, or gross pathology.

Microscopic findings were observed in the epididymides and testes of the 0.20 mg/L animals, as well as the control animals, but these changes were considered unrelated to 2,4-DB administration because they occurred with generally similar incidence and severity in the 0.20 mg/L and control groups. In addition, these effects were similar to changes that have been noted and attributed to thermal stress and immobilization of rats confined in inhalation tubes in previous studies.

The systemic LOAEC was not determined. The systemic NOAEC is 0.20 mg/L.

Microscopic changes in the larynx (squamous metaplasia of the laryngeal respiratory and epithelium inflammation of the adjacent connective tissue) were observed at ≥0.02 mg/L after inhalation exposure to 2,4-DB for four weeks. Full recovery of the squamous metaplasia was achieved within four weeks after cessation of exposure to 2,4-DB, with partial recovery achieved for inflammation.

An expert international workshop on laryngeal squamous metaplasia in rodent studies has concluded that laryngeal epithelial squamous metaplasia may be accompanied by submucosal inflammation dependent on the irritant effect of the compound. Inflammatory responses due to irritant effects are regarded as adverse (Kaufmann *et al*., 2009[1]). At the highest dose tested, 0.20 mg/L, an increase in severity score for laryngeal epithelial squamous metaplasia and inflammation occurred. At 0.05 mg/L the severity of squamous metaplasia was "minimal-slight" while at 0.20 mg/L the scoring was "slight-moderate". All animals at 0.05 mg/L had an inflammation score of "minimal" as compared "minimal-slight" at 0.20 mg/L. The highest dose tested was considered adverse due to the increase in severity score to include "slight-moderate" squamous metaplasia in combination with inflammation.

The inhalation LOAEC 0.20 mg/L based on portal-of-entry laryngeal epithelial squamous metaplasia and inflammation. The inhalation NOAEC is 0.05 mg/L.

This study is classified **acceptable / guideline** and satisfies the guideline requirements of OPPTS 870.3465 in the rat.

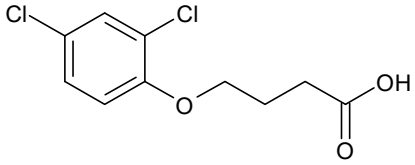
COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided. It was stated that the study was conducted in compliance with the US EPA's Good Laboratory Practice Regulations (Part 160 of 40 CFR-FIFRA), with one exception: the anatomic pathologist provided representative photomicrographs of the target organs in each test group at the end of the treatment and recovery periods to demonstrate the progression or recovery of lesions. These photomicrographs were for illustrative purposes only and were not generated in compliance with the referenced GLP regulation. This exception was not considered to affect the integrity or validity of the study because the information was derived by the expertise of the anatomic pathologist.

1 Kaufmann, W.; Bader, R.; Ernst, H. et al. (2009) 1st international ESTP expert workshop: "larynx squamous metaplasia". A re-consideration of morphologic and diagnostic approaches in rodent studies and its relevance for human assessment. *Exp. Toxicol. Pathol.* 61: 591-603.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: 2,4-DB technical acid

Description:	Off-white solid
Lot #:	80006961
Purity:	97.74 % a.i.
Compound stability:	Not reported
CAS # of TGAI:	94-82-6
Structure:	

2. Vehicle: Air only

3. Test animals

Species:	Rat
Strain:	Hsd:Sprague Dawley (SD)
Age/weight at study initiation:	Approximately nine weeks / 230-311 g (males only)
Source:	Envigo (Frederick, MD)
Housing:	2-3 rats/sex in polycarbonate cages with stainless steel-mesh lids and cellulose bedding.
Diet:	Teklad Global 16% Protein Rodent Diet (Certified), 2016C (Envigo, Madison, WI) *ad libitum* (except during exposure and overnight prior to blood sampling for clinical pathology and necropsy)
Water:	Tap water, *ad libitum* (except during exposure)
Environmental conditions:	**Temperature:** 20-26°C **Humidity:** 30-70% **Air changes:** Not provided **Photoperiod:** 12 h light / 12 h dark
Acclimation period:	Approximately three weeks. Animals were acclimated to the method of restraint over a 7 day period immediately preceeding the first exposure.

B. STUDY DESIGN

1. In-life dates: Start: December 11, 2017 End: February 3, 2018

2. Animal assignment: The rats were assigned to the test groups noted in Table 1a by random allocation. Mean achieved gravimetric data are provided in Table 1a and mean achieved analytical data are provided in Table 1b. It was stated that the individual body weights at allocation were within ±20% of the mean. Ten animals/concentration were assigned to the terminal necropsy groups (after four weeks of administration), and six animals/concentration/week were assigned to the recovery necropsy groups after Weeks 1, 2, and 4 of recovery.

TABLE 1a: Study design and mean achieved gravimetric data for all treatment groups [a]						
Test group	**Nominal conc. (mg/L)**	**Gravimetric conc. (mg/L)**	**Gravimetric MMAD (µm)**	**Gravimetric GSD**	**Terminal necropsy**	**Recovery necropsy [b]**
Control	0	0.000	---	---	10	6/week
Low	0.02	0.023	2.4	1.66-2.67	10	6/week
Mid	0.05	0.056	2.0	1.65-2.19	10	6/week
High	0.20	0.230	2.3	1.70-2.07	10	6/week

a Data were obtained from pages 22 and 41 of MRID 50624901.
b Recovery Weeks 1, 2, and 4.

TABLE 1b: Mean achieved analytical data for all treatment groups [a]				
Test group	**Nominal conc. (mg/L)**	**Analytical conc. (mg/L)**	**Analytical MMAD (µm)**	**Analytical GSD**
Control	0	0.000	---	---
Low	0.02	0.020	2.1	1.91-1.98
Mid	0.05	0.057	2.0	1.88-1.95
High	0.20	0.233	2.3	1.93-2.06

a Data were obtained from page 41 of MRID 50624901.

3. **Dose selection rationale:** It was stated that the concentration selection rationale for the 28-day inhalation toxicity study was based on a preliminary three-week inhalation study (Envigo Study No. PW59VN[2]) that was conducted at target exposure levels of 0, 0.02, 0.05, and 0.20 mg/L to assess the toxicity and toxicokinetic profile of 2,4-DB in rats after administration by nose-only inhalation.

   The results of the preliminary investigation demonstrated no systemic toxicity, but did show portal-of-entry effects at ≥0.02 mg/L that were considered not adverse by the conducting laboratory (epithelial alteration and squamous metaplasia in the larynx and mucous cell hyperplasia in the nose/turbinates). Based on this evaluation, the same exposure levels (0, 0.02, 0.05, and 0.20 mg/L) were selected for the definitive 28-day inhalation study. The test was conducted in males only because prior non-inhalation studies had shown males to be the more sensitive sex.

4. **Generation of the test atmosphere / chamber description:** Diagrams of the exposure systems for air control and test substance inhalation were provided on pages 403 and 404 of MRID 50624901, and are included at the end of this DER as Appendices 1 and 2, respectively.

   Exposures were conducted with ADG (40-L) nose-only exposure systems with two levels (20 animal ports/level) for each system. The rats were restrained at each exposure port with molded, clear thermoplastic tubes, and unused ports were sealed with stoppers. Individual exposure systems were dedicated to each dose group for the duration of the study. A compressed, in-house, breathing-quality air supply was connected directly to the exposure system inlet for the air control group. For the treatment groups, the air supply and the aerosol generation device were connected with corrugated tubing to a thermoplastic expansion chamber located at the top of each system. The inlet generation airflow consisted of dry air from an in-house, compressed air system. Airflow for the control group was 28 L/min, and 20 L/min for the treatment groups. The dilution airflow also consisted of dry air from the in-house, compressed air system, with a flow rate of 8 L/min for each treatment

2 Hoffman, G. 2,4-DB technical acid: a preliminary toxicity study by inhalation administration to rats for 3 weeks with a 1 week recovery period. Envigo study No. PW59VN.

system. Additionally, 2 L/min of room airflow was drawn passively into each chamber by a tangential inlet port for all dose groups. All exhaust airflow was drawn out at the base of each exposure system by an in-house extract system at a rate of 30 L/min per system through HEPA-filtration. In-line, variable air flowmeters were used to monitor airflows continuously.

Chamber temperature and relative humidity were monitored continuously, and documented at approximately 30-min intervals. The mean temperature and mean relative humidity ranged from 20-22°C and 49-53%, respectively. Chamber CO_2 concentration samples were determined at least once per study/exposure system. Measured CO_2 concentrations ranged from 0.7-0.9%.

An aerosol atmosphere of the test substance was generated as follows. A Mark I Wright Dust Feeder was connected prior to the generation air supply for each treatment group exposure system. Test aerosol concentrations were achieved by alterations of the canister diameter and the advance rate. The test aerosol entered the expansion chamber where it was combined with dilution air as necessary. The resulting test substance atmosphere was delivered to the expansion chamber at the top of each exposure system.

Test atmosphere concentration: The achieved gravimetric and analytical (HPLC/UV) concentrations are reported in Tables 1a and 1b, respectively. Samples were collected on glass-fiber filters that were held in open-face filter holders. The sample flow rate was 2.0 L/min, and was measured with a timed flowmeter. Samples were collected from the approximate animal-breathing zone of each exposure system. A minimum of three samples/dose group were collected during each exposure. Filters collected on Exposure Days 1-4, and weekly thereafter, were sent assessed for concentration analysis. Filters that were not analyzed were stored refrigerated at (2-8°C).

Particle size determination: Particle size results are presented in Tables 1a and 1b. Aerosol particle size determinations were conducted by using a cascade impactor with stainless steel discs as collection substrates for the initial stages, and a glass-fiber filter as the final collection substrate. Aerosol particle size measurements were conducted at a minimum of one sample/week for each test substance group from the approximate animal-breathing zone of each exposure system. Samples were collected at a flow rate of 2.0 L/min, and sample volume was determined by a wet-type gas meter. The stainless steel collection substrates and the final-stage filters were assessed. The particle size was expressed as the mass mean aerodynamic diameter (MMAD) in microns and the geometric standard deviation (GSD), and were determined by gravimetric and HPLC/UV methods. The determined MMAD values for all samples collected demonstrated that the delivered particle sizes were respirable for the rats.

5. **Exposure schedule:** The rats were exposed to the 2,4-DB aerosol by nose-only inhalation for six h/day, five days/week through the day prior to scheduled euthanasia for a total of 20 days. Animal positions of the exposure system levels were changed weekly. At the end of each 6-h exposure period, the rats remained in the exposure chambers for a minimum 7-min chamber clear-out period. Animals were returned to their home cages between exposures. The first day of exposure was designated as Day 1.

6. Statistics: All statistical analyses were carried out with the individual animal as the basic experimental unit. Body weight, body weight gain (interval and cumulative from baseline), food consumption, hematology/coagulation, clinical chemistry, urinalysis, and organ weight data were analyzed separately for each time point. Prior to statistical analysis, parameters were identified as continuous, discrete, or binary.

Continuous parameters: If Bartlett's test for homogeneity of variance was not significant at the 1% level, a parametric method was applied. If the F1 approximate test for monotonicity of exposure-response was not significant at the 1% level, a two-tailed Williams' test for a monotonic trend was applied. If the F1 test was significant, a two-tailed Dunnett's test was conducted instead. If Bartlett's test was significant at the 1% level, logarithmic and square-root transformations were attempted. If Bartlett's test was still significant, a non-parametric method was applied to mean ranks. If the H1 approximate test for monotonicity was not significant at the 1% level, a two-tailed Shirley's test for a monotonic trend was applied. If the H1 test was significant, a two-tailed Steel's test was conducted instead.

For pre-treatment data, if Bartlett's test for homogeneity of variance was not significant at the 1% level, a parametric method was applied. Analysis of variance was used to test for any group differences. If the test was significant at the 5% level, inter-group comparisons with two-tailed t-tests, with the error mean square from the one-way analysis of variance, were made. If Bartlett's test was significant at the 1% level, logarithmic and square-root transformations were attempted. If Bartlett's test was still significant, a non-parametric method was applied to mean ranks. Kruskal-Wallis' test was used to test for any group differences. If the test was significant at the 5% level, inter-group comparisons were made with two-tailed Wilcoxon rank sum tests.

Discrete parameters: If the two-tailed Jonckheere-Terpstra test was significant at the 5% level, the direction of the trend was established and one-tailed, step-down testing in this direction was conducted. If the Jonckheere-Terpstra test was not significant at the 5% level, the Kruskal-Wallis test was applied. If the Kruskal-Wallis test was significant at the 5% level, the treated groups were compared to control by using exact two-tailed Wilcoxon rank sum tests. Otherwise, no further comparisons were made. For pre-treatment data, a Kruskal-Wallis test was conducted. If the test was significant at the 5% level, treated groups were compared to control with exact two-tailed Wilcoxon rank sum tests.

Binary parameters: For comparisons involving more than two groups, if the two-tailed Cochran-Armitage test was significant at the 5% level, the direction of the trend was established and one-tailed, step-down testing in this direction was conducted. If the Cochran-Armitage test was not significant at the 5% level, a χ^2 test was applied. If the χ^2 test was significant at the 5% level, the treated groups were compared to control with two-tailed Fisher's Exact test. Otherwise, no further comparisons were made. For pre-treatment data, an exact χ^2 test was conducted. If the test was significant at the 5% level, treated groups were compared to control by using two-tailed Fisher's Exact test. For pre-treatment data, an exact χ^2 test was performed. If the test was significant at the 5% level, treated groups were compared to control by using two-tailed Fisher's Exact tests.

These analyses were considered appropriate by the reviewers.

C. METHODS

1. Observations

a. Cageside observations: The rats were observed in their cages twice daily for mortality and assessment of general condition. Animals were observed prior to and after test substance administration (~1 hour - examined by group) on each exposure day.

b. Clinical examinations: Detailed clinical examinations were conducted twice prior to the initiation of treatment and weekly during the study period (after exposure on exposure days). Examinations included observation of general condition, skin and fur, eyes, nose, oral cavity, abdomen, and external genitalia, as well as an evaluation of respiration.

Body temperatures were determined with rectal probes for five animals/dose group within 1 hour after the initial exposure period and after an exposure during Week 4 (within 1 hour of exposure).

c. Neurological evaluations: Neurological examinations were not conducted.

2. Body weight: Individual, non-fasted body weights were recorded twice prior to the initiation of treatment and twice weekly during the exposure period (prior to exposure on exposure days). Body weights were recorded weekly during the recovery periods. Body weights were reported for pretreatment Days 10 and 17; Days 1, 5, 8, 12, 15, 19, 22, and 26; and recovery Days 6, 13, 20, and 27, with interval body weight gains and cumulative body weight gains reported for each interval. Fasted, terminal body weights were recorded on the day of scheduled euthanasia prior to necropsy.

3. Food consumption: Food consumption (g/rat/day) was determined by weight every seven days starting one week prior to the initiation of treatment.

4. Ophthalmoscopic examination: Ophthalmoscopic examinations were conducted on all rats prior to the initiation of treatment and during Week 4 of exposure. Eyelids, lacrimal apparatus, and conjunctiva were examined visually. The cornea, anterior chamber, lens, iris, vitreous humor, retina, and optic disc were examined with an indirect ophthalmoscope, after pupillary dilation with a mydriatic agent.

5. Hematology and clinical chemistry: Blood was collected on Day 27 from ten rats/dose group following an overnight fast. Blood for hematology/coagulation and clinical chemistry analyses was collected at euthanasia from the vena cava of rats anesthetized with isoflurane as a terminal procedure. The CHECKED (X) parameters were examined.

a. Hematology

X	Hematocrit (HCT)*	X	Leukocyte differential count*
X	Hemoglobin (HGB)*	X	Mean corpuscular HGB (MCH)*
X	Leukocyte count (WBC)*	X	Mean corpuscular HGB conc.(MCHC)*
X	Erythrocyte count (RBC)*	X	Mean corpuscular volume (MCV)*
X	Platelet count*	X	Reticulocyte count
	Blood clotting measurements*	X	Red cell distribution width
X	(Activated partial thromboplastin time)		
	(Clotting time)		
X	(Prothrombin time)		

* Recommended for subchronic inhalation studies based on Guideline 870.3465.

It was stated that a peripheral blood smear was prepared for each animal, and that the smears were available for confirmation of automated results and/or other evaluations if necessary. Also, manual differential WBC counts were conducted for verification, and absolute values were calculated, if necessary.

b. Clinical chemistry

	ELECTROLYTES		OTHER
X	Calcium	X	Albumin*
X	Chloride	X	Creatinine*
	Magnesium	X	Urea nitrogen*
X	Phosphorus	X	Total cholesterol*
X	Potassium*	X	Globulins
X	Sodium*	X	Glucose*
	ENZYMES (more than 2 hepatic enzymes eg., *)	X	Total bilirubin
X	Alkaline phosphatase*	X	Total serum protein (TP)*
	Cholinesterase (ChE)	X	Triglycerides
	Creatine phosphokinase		Serum protein electrophoresis
	Lactic acid dehydrogenase (LDH)	X	A/G ratio
X	Alanine aminotransferase (ALT/also SGPT)*		Appearance
X	Aspartate aminotransferase (AST/also SGOT)*		
	Sorbitol dehydrogenase*		
	Gamma glutamyltransferase (GGT)*		
	Glutamate dehydrogenase		

* Recommended for subchronic inhalation studies based on Guideline 870.3465.

6. **Urinalysis**: Urine was collected during a 12- to 16-hour overnight collection (night of Day 28) using metabolism cages from ten rats/dose group. Animals had access to water *ad libitum*, but no access to food during the collection period. The CHECKED (X) parameters were examined.

X	Appearance*	X	Glucose*
X	Volume*	X	Ketones
X	Specific gravity / osmolality*	X	Bilirubin
X	pH*	X	Blood / blood cells*
	Sediment (microscopic)		Nitrate
X	Protein*		Urobilinogen

* Optional for inhalation toxicity studies based on Guideline 870.3465

7. **Sacrifice and pathology:** Macroscopic examinations were performed on all aniamls and included external and detailed internal examinations. Necropsies were conducted on ten animals/dose group after animals had been treated for four weeks, and on six animals/dose

group after recovery for one, two, or four weeks after the end of the exposure period. Animals were fasted overnight prior to necropsy. All rats were euthanized by exsanguination under isoflurane anesthesia and subjected to a complete gross pathological examination. The CHECKED (X) tissues were collected for histological examination, and the (XX) organs were weighed (paired organs weighed together).

	DIGESTIVE SYSTEM		CARDIOVASC./HEMAT.		NEUROLOGIC
X	Tongue	X	Aorta, thoracic*	XX	Brain*+
X	Salivary glands*	XX	Heart*+	X	Peripheral nerve (sciatic)*
X	Esophagus*	X	Bone marrow (femoral and sternal)*	X	Spinal cord (3 levels)*
X	Stomach*	X	Lymph nodes*	XX	Pituitary* [c]
X	Duodenum*	XX	Spleen*+	X	Eyes (optic nerve)*
X	Jejunum*	XX	Thymus*+		**GLANDULAR**
X	Ileum*			XX	Adrenal gland*+
X	Cecum*		**UROGENITAL**	X	Lacrimal gland
X	Colon*	XX	Kidneys*+	XX	Parathyroid* [c, d]
X	Rectum*	X	Urinary bladder*	XX	Thyroid* [c, d]
XX	Liver*+	XX	Testes*+		**OTHER**
	Gall bladder* (not rat)	XX	Epididymides*+	X	Bone (sternum and femur)
	Bile duct* (rat)	XX	Prostate* [a]	X	Skeletal muscle
X	Pancreas*	XX	Seminal vesicles* [a]	X	Skin
	RESPIRATORY		Ovaries (w/oviducts)*+	X	All gross lesions/masses*
X	Trachea*		Uterus*+	X	Peyer's patches/GALT [b]
XX	Lung (w/bronchi)*	X	Mammary gland* [b]	X	Harderian glands
X	Nasal cavity (w/turbinates)*		Cervix	X	Zymbal's gland
X	Pharynx*		Vagina		
X	Larynx*				

* Recommended for subchronic rodent studies based on Guideline 870.3465
\+ Organ weights required
a Weighed together.
b Mammary gland and GALT evaluated if present in routine sections.
c Weights determined post-fixation.
d Evaluation of single organ sufficient if one of pair is missing.

Eyes and testes were placed in modified Davidson's solution, followed by 10% neutral buffered formalin (NBF). Lungs were infused with 10% NBF prior to immersion in the same fixative. All other tissues were preserved in 10% NBF.

After fixation, tissues and organs from all animals were processed routinely, embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin. All tissues from the control and 0.20 mg/L groups were examined microscopically with the following exceptions: the larynx was examined in all groups and in all recovery periods; all gross lesions were examined; and the optic nerve, rectum, tongue, and Zymbal's gland were not examined in any group. Bones were decalcified in Formical-2000.

In addition, six levels of each larynx were obtained following departmental guidelines. Three anatomic levels of the larynx were evaluated per Registry of Industrial Toxicology Animal-data (RITA) Revised Guides for Organ Sampling and Trimming in Rats and Mice. Level I sampled the anterior larynx at the base of the epiglottis and included the seromucinous glands. Level II sampled the mid-larynx and included the ventral pouch, u-shaped cartilage and arytenoids. Level II sampled the posterior larynx and included the cricoid cartilage (Appendix 3). The three levels were examined microscopically utilizing a total of six tissue sections; three tissue sections from

Level 1, one tissue section from Level 2 and two tissue sections from Level 3. This was done to ensure adequate visualization of all relevant structures. Microscopic findings within a given tissue section were recorded for the anatomic level in which they occurred. Six anatomic levels of the nasal cavity were collected according to the Hamnar method and were evaluated microscopically (NEIHS 2012[3]).

Histopatholgy was graded according to the following scale:

Minimal: The change is barely discernible and/or very few/very small foci or areas are affected.
Slight: The change is more noticeable but only evident as few/small foci or areas affected.
Moderate: The change is obviously present, and of appreciable size and/or number.
Marked: The change is abundant in many areas of the section and/or is of prominent size.
Severe: The change affects a large proportion of the tissue and/or is of a large size.

II. RESULTS

A. OBSERVATIONS

1. Mortality: One 0.02 mg/L male was euthanized on Day 8 due to skin lesions (macroscopic periocular scabbing that correlated with microscopic findings of marked ulceration and moderate pyogranulomatous inflammation). These findings were not considered related to treatment with 2,4-DB because they occurred in a single animal and were unrelated to dose. All other rats survived to scheduled euthanasia.

2. Clinical signs of toxicity: There were no treatment-related effects on clinical signs or on body temperatures. Group mean body temperatures ranged from 37.8-38.4°C on Day 1 and 38.5-38.8°C on Day 24.

B. BODY WEIGHT AND WEIGHT GAIN: Body weight and body weight gain data are presented in Table 2. There were no treatment-related effects on body weight or body weight gain.

TABLE 2. Mean (±SD) body weights and body weight gains (g) in male rats treated with 2,4-DB by nose-only inhalation for four weeks, with up to a four-week recovery period. [a]

Day	Concentration (mg/L)			
	0	0.02	0.05	0.20
1 [b]	274 ± 12.2	274 ± 16.0	270 ±9.9	272 ± 12.7
8 [b]	290 ± 13.4	291 ± 19.9	287 ± 12.4	286 ± 15.4
15 [b]	304 ± 15.7	305 ± 23.7	301 ± 13.2	300 ± 16.5
22 [b]	323 ± 16.1	324 ± 29.6	320 ± 13.8	318 ± 17.7
26 [b]	326 ± 17.4	329 ± 27.1	323 ± 15.7	321 ± 19.2
R6 [c]	346 ± 17.5	348 ± 23.5	344 ± 15.1	336 ± 18.7
R13 [d]	358 ± 21.8	359 ± 16.3	359 ± 15.8	348 ± 17.5
R20 [e]	384 ± 29.0	390 ± 17.8	372 ± 18.2	376 ± 12.7
R27 [e]	397 ± 32.3	401 ± 18.4	382 ± 19.0	385 ± 12.6
BWG 1-26 [b]	52 ± 7.9	55 ± 13.2	53 ± 8.1	49 ± 9.8
BWG 26-R27 [f]	71	72	59	64
BWG Day 1-R27 [e]	123 ± 22.9	131 ± 8.4	112 ± 13.9	116 ± 10.3

a Data obtained from Table 5 on pages 58-60 of MRID 50624901.

3 NEIHS, EPL, NTP (June 2012). Rodent Respiratory Tissue Collection Protocol. CD-ROM

b n = 28
c n = 18
d n = 12
e n = 6
f Calculated by the reviewers as the difference between mean body weight on Days R27 and 26.

C. FOOD CONSUMPTION: There were no treatment-related effects on food consumption. Although a decrease ($p<0.05$) in food consumption was determined for the 0.20 mg/L group for Day 1-8 (17 ± 0.9 g/animal/day treated vs. 18 ± 1.2 g/animal/day control), this minor change was not considered related to treatment or adverse. Differences of the same magnitude were observed throughout the treatment period with no relation to dose or direction.

D. OPHTHALMOSCOPIC EXAMINATIONS: There were no treatment-related effects observed during the ophthalmoscopic examinations.

E. BLOOD ANALYSES

1. Hematology: There were no treatment-related effects noted on any hematology/coagulation parameters. Decreases ($p<0.05$) in prothrombin time values for the 0.05 and 0.20 mg/L groups (0.8 sec and 0.7 sec, respectively) were not considered related to treatment because there was no dose dependency and the direction of the change is not considered adverse. Similar decreases (not significant [NS]) noted in activated partial thromboplastin time values for the 0.02, 0.05, and 0.20 mg/L groups (0.8 sec, 1.7 sec, and 1.2 sec, respectively) also were not considered related to treatment or adverse. Hematology/coagulation assessments were not conducted after the 4-week recovery period.

2. Clinical chemistry: Clinical chemistry parameter data are presented in Table 3. Clinical chemistry changes that occurred at 0.20 mg/L included increased ($p<0.05$) alanine aminotransferase (↑17%), increased (NS) alkaline phosphatase (↑14%), decreased ($p<0.01$) total cholesterol (↓15%), and minor increases ($p<0.05$) in sodium and chloride ions and calcium concentrations (↑1-5%). These minor changes did not correlate with an overall pattern of toxicity in clinical pathology parameters, and had no correlates in macroscopic or microscopic pathology. Changes at <0.20 mg/L were either NS or unrelated to dose, and were considered unrelated to treatment. Clinical chemistry assessments were not conducted after the 4-week recovery period.

TABLE 3. Mean (±SD) clinical chemistry parameter data in male rats treated with 2,4-DB by nose-only inhalation for four weeks [a]

Parameter	Concentration (mg/L)			
	0	0.02	0.05	0.20
Alanine aminotransferase (U/L)	46 ± 7.6	48 ± 6.4	50 ± 6.6 (↑9%)	54 ± 6.3* (↑17%)
Alkaline phosphatase (U/L)	103 ± 15.4	110 ± 13.6	110 ± 19.2	117 ± 14.7 (↑14%)
Cholesterol (mg/dL)	95 ± 11.8	97 ± 10.6	85 ± 10.8 (↓11%)	81 ± 6.9** (↓15%)
Sodium (mEq/L)	140 ± 1.8	142 ± 1.4	140 ± 1.1	142 ± 1.1** (↑1%)
Chloride (mEq/L)	101 ± 1.7	102 ± 1.3	101 ± 1.3	103 ± 1.4* (↑2%)
Calcium (mg/dL)	10.0 ± 0.20	10.3 ± 0.29* (↑3%)	10.7 ± 0.28** (↑7%)	10.5 ± 0.47** (↑5%)

a Data obtained from Table 10 on pages 66-67 of MRID 50624901; n=10. Percent differences from control (calculated by the reviewers) are included in parentheses.
* Significantly different from the control; $p<0.05$.
** Significantly different from the control; $p<0.01$.

F. URINALYSIS: There were no treatment-related effects observed on any urinalysis parameters. Decreases ($p<0.05$) in pH values noted at ≥0.05 mg/L were minor and unrelated to dose.

G. SACRIFICE AND PATHOLOGY

1. Organ weight: There were no organ weight changes in the terminal necropsy animals (Day 27) or in any animals after recovery at Weeks 1, 2, and 4. Sporadic differences in relative (to body) or relative (to brain) organ weighs were observed, but were considered related to treatment due to a lack of dose dependence and/or macroscopic/microscopic correlates.

2. Gross pathology: There were no treatment-related findings observed at the terminal or recovery necropsies.

3. Microscopic pathology: Selected microscopic findings at terminal necropsy (Day 27) are presented in Table 4. Minimal to moderate squamous metaplasia of the laryngeal respiratory epithelium, and minimal to slight inflammation, occurred at ≥0.02 mg/L. These changes were located at the level of the anterior larynx (Level I) at or near the base of the epiglottis, and were dose-related in incidence and/or severity. Squamous metaplasia was characterized by five or more layers of flattened epithelial cells, with keratinization and desquamation of superficial keratinocytes occurring with increases in severity. Inflammation was observed in the connective tissue immediately subjacent to the metaplastic epithelium. Inflammation was characterized by granulation tissue infiltrated by lymphocytes, plasma cells, macrophages, and neutrophils (rare).The granulation tissue was composed of increased fibroblasts, increased small caliber blood vessels, and slightly more prominent extracellular collagen fibers.

Microscopic findings were observed in the epididymides and testes of the 0.20 mg/L animals, as well as the control animals. The epididymides had slight to marked increases in cell debris (9/10 treated vs. 10/10 control) and slight to severe reduction of sperm (8/10 treated vs. 7/10 control) at 0.20 mg/L. The cell debris consisted of degenerated

spermatids, with concomitant reductions of sperm in the epididymal ducts (particularly the cauda epididymis). The testes had minimal to moderate tubular degeneration/atrophy (9/10 treated vs. 10/10 control) and spermatid degeneration/depletion (10/10 treated vs. 8/10 control) at 0.20 mg/L. Changes in the testes included reduced numbers of spermatids, degeneration and sloughing of elongating spermatids, and spermatid retention. Tubular vacuolation also was noted, in addition to focal or partial depletion of germ cells. One epididymis or testis was frequently affected more severely relative to the other. These changes were considered unrelated to 2,4-DB administration because they occurred with generally similar incidence and severity relative to control. In addition, these effects were similar to changes caused by thermal stress and immobilization of rats in inhalation tubes (Lee *et al*., 1993[4] and Everds *et al*., 2013[5]).

TABLE 4. Selected microscopic findings in male rats treated with 2,4-DB by nose-only inhalation for four weeks. [a]

Organ		Concentration (mg/L)			
		0	**0.02**	**0.05**	**0.20**
Larynx (Level I)					
Number of tissues examined		**9**	**9** [b]	**10**	**10**
Metaplasia, squamous, respiratory epithelium	Minimal	0	8	4	0
	Slight	0	1	6	8
	Moderate	0	0	0	2
	Total	0	9	10	10
Inflammation	Minimal	0	8	10	8
	Slight	0	0	0	2
	Total	0	8	10	10
Larynx (Level II)					
Dialted Gland, in floor of ventral pouch	Slight	0	0	1	0
	Total	0	0	1	0
Larynx (Level III)					
No findings					
Epididymides					
Number of tissues examined		**10**	**0**	**0**	**10**
Cell debris, luminal	Slight	4	---	---	3
	Moderate	5	---	---	3
	Marked	1	---	---	3
	Total	10			9
Sperm, reduced, luminal	Slight	3	---	---	3
	Moderate	1	---	---	3
	Marked	3	---	---	0
	Severe	0	---	---	2
	Total	7			8
Testes					

4 Lee, K.; Frame, S.R.; Sykes, G.P.; and Valentien, R. (1993) Testicular Degeneration and Spermatid Retention in Young Male Rats. *Toxicol. Pathol.* 21:292-302.

5 Everds, N.E.; Snyder, P.W.; Bailey, K.L.; Bolon, B.; Creasy, D.M.; Foley, G.L.; Rosol, T.J.; and Sellers, T. (2013) Interpreting Stress Responses during Routine Toxicity Studies: A Review of the Biology, Impact, and Assessment *Toxicol. Pathol.* 4:560-614.

Number of tissues examined		**10**	**0**	**0**	**10**
Degeneration/atrophy, tubular	Minimal	6	---	---	4
	Slight	3	---	---	2
	Moderate	1	---	---	3
	Total	10	---	---	9
Spermatid degeneration/depletion	Minimal	5	---	---	7
	Slight	2	---	---	2
	Moderate	1	---	---	1
	Total	8			10
Nose/Turbinates					
Number of tissues examined		**10**	**0**	**0**	**10**
Infiltrate, inflammatory cell, mononuclear	Minimal	0	---	---	2
	Total	0	---	---	2

a Data obtained from Table 16 on pages 98-107 of MRID 50624901.
b Inclusion of the single animal euthanized on Day 8 does not alter these laryngeal findings.

Selected microscopic findings during the 4-week recovery period are presented in Table 5. During the four-week recovery period, laryngeal squamous metaplasia of the anterior larynx (Level I) recovered completely, but inflammation recovered only partially. Squamous metaplasia of the laryngeal respiratory epithelium had recovered completely at 0.02 mg/L after one week of recovery, but recovered completely at 0.05 and 0.20 mg/L after recovery for four weeks. Inflammation associated with squamous metaplasia recovered partially, with severity decreased to minimal after one week of recovery, but inflammation was still observed at ≥0.02 mg/L after four weeks of recovery.

TABLE 5. Selected microscopic findings in male rats treated with 2,4-DB by nose-only inhalation for four weeks and allowed a 4-week recovery period. [a]

Organ		**Concentration (mg/L)**			
		0	**0.02**	**0.05**	**0.20**
Larynx (Level I) – Recovery Week 1					
Number of tissues examined		**6**	**6**	**6**	**6**
Metaplasia, squamous, respiratory epithelium	Minimal	0	0	1	6
	Total	0	0	1	6
Inflammation	Minimal	0	1	3	6
	Total	0	1	3	6
Larynx (Level II) – Recovery Week 1					
No findings					
Larynx (Level III) – Recovery Week 1					
Multnucleated giant cells and/or mineralized secreations	Minimal	0	0	1	1
	Total	0	0	1	1
Larynx (Level I) – Recovery Week 2					
Number of tissues examined		**6**	**6**	**6**	**6**
Metaplasia, squamous, respiratory epithelium	Minimal	0	0	1	4
	Total	0	0	1	4
Inflammation	Minimal	0	2	6	5
	Total	0	2	6	5
Larynx (Level II) – Recovery Week 2					
Dilated Gland	Slight	0	0	1	0
	Total	0	0	1	0
Larynx (Level III) – Recovery Week 2					
Multnucleated giant cells and/or mineralized secreations	Minimal	1	1	1	1
	Total	1	1	1	1

Larynx (Level I) – Recovery Week 4					
Number of tissues examined		**6**	**6**	**4**	**6**
Inflammation	Minimal	0	2	3	4
	Total	0	2	3	4
Larynx (Level II) – Recovery Week 4					
Dilated Gland	Minimal	0	0	1	0
	Total	0	0	1	0
Larynx (Level III) – Recovery Week 4					
Multnucleated giant cells and/or mineralized secreations Minimal		0	0	0	1
	Total	0	0	0	1

a Data obtained from Table 16 on pages 108-112 of MRID 50624901.

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATORS CONCLUSIONS</u>: Inhalation exposures of 2,4-DB Technical Acid to rats for four weeks (five days per week for six hour per session) at target exposure levels of 0.02, 0.05, and 0.20 mg/L resulted in no test item-related effects on clinical signs, body weights, food consumption, or clinical pathology parameters.

Four-weeks repeat inhalation exposure to 2,4-DB Technical Acid was associated with minimal to moderate squamous metaplasia and minimal to slight inflammation of the anterior larynx at ≥0.02 mg/L. Squamous metaplasia of the laryngeal respiratory epithelium recovered completely at 0.02 mg/L after a one-week recovery period, and recovered completely at 0.05 and 0.2 mg/L after a four-week recovery period. Inflammation associated with squamous metaplasia was considered to have recovered partially. The severity decreased to minimal after a one-week recovery period, but after a four-week recovery period minimal inflammation was still observed in a few animals. Laryngeal squamous metaplasia is considered an adaptive non-specific response to chronic irritation by which a susceptible epithelium is replaced by a more resistant one. Such induced changes in rodents usually fail to result in the development of lesions in primates and are not considered indicative of significant risk in humans (Lewis, 1991[6]; Osimitz *et al*., 2007[7]). In this study, squamous metaplasia was accompanied by submucosal inflammation at ≥0.02 mg/L. An expert international workshop on laryngeal squamous metaplasia in rodent studies has concluded that laryngeal epithelial squamous metaplasia may be accompanied by submucosal inflammation dependent on the irritant effect of the compound. Inflammatory responses due to irritant effects are regarded as adverse (Kaufmann *et al*., 2009[8]). Per recommendations of the Society of Toxicologic Pathology Scientific and Regulatory Committee, the assessment of adversity applies only to this species under the conditions of this study (Kerlin *et al*., 2016[9]).

6 Lewis, D.J. (1991) Morphological assessment of pathological changes within the rat larynx. *Toxicol. Pathol.* 19:352-357.
7 Osimitz, T.G.; Droege, W.; and Finch, J.M. (2007) Toxicologic Significance of Histologic Change in the Larynx of the Rat Following Inhalation Exposure: A Critical Review. *Toxicol Appl Pharmacol.* 225:229-237.
8 Kaufmann, W.; Bader, R.; Ernst, H. et al. (2009) 1st international ESTP expert workshop: "larynx squamous metaplasia". A re-consideration of morphologic and diagnostic approaches in rodent studies and its relevance for human assessment. *Exp. Toxicol. Pathol.* 61: 591-603.
9 Kerlin, R.; Bolon. B.; Burkhardt, J.; Francke, S.; Greaves, P.; Meador, V.; and Popp, J. (2016) Scientific and regulatory policy committee: Recommendations ("best") practices for determining, communicating and using adverse effects data from nonclinical studies. *J. Toxicol Pathol.* 44(2):147-162.

Testicular degeneration and reduced sperm density and/or sloughed germ cells in the epididymides were observed in almost all control and 0.20 mg/L males. These changes have been reported in association with restraint in inhalation tubes, and have been attributed to immobilization and thermal stress. The difference in severity between pairs of testes and epididymides in this study suggests that one testis may have become warmer than the other during exposures, possibly because of asymmetrical positioning relative to the body and/or the wall of the inhalation tube. The high incidence and severity of the testicular changes observed in this study were unexpected based on the sporadic and minimal changes seen in previous studies carried out at these laboratories using nose-only inhalation for a similar duration. However, previous studies of this duration have only been performed at these laboratories in Crl SD rats, so the increased incidence and severity of the testicular lesions in the Harlan SD rats were considered to be due to a strain difference in susceptibility to restraint-induced immobilization/heat stress. Since the testicular findings were similar in control and test article-treated rats, they were considered to be related to the restraining procedure used during the inhalation exposure and not related to 2,4-DB Technical Acid.

In conclusion, under the conditions of this study and based on the histopathology findings, the No Observed Effect Level (NOEL) and the No Observed Adverse Effect Level (NOAEL) were not determined for portal-of-entry toxicity. However, under the conditions of this study and based on all other findings, the No Observed Adverse Effect Level (NOAEL) was considered to be the high exposure level of 0.20 mg/L for systemic toxicity.

B. REVIEWER COMMENTS: There were no effects of treatment on mortality, clinical signs, body weight/body weight gain, food consumption, ophthalmoscopic examinations, hematology, clinical chemistry, urinalysis, organ weights, or gross pathology.

Microscopic findings were observed in the epididymides and testes of the 0.20 mg/L animals, as well as the control animals. These changes included increases in cell debris and reduction of sperm in the epididymides, and tubular degeneration/atrophy, spermatid degeneration/depletion, and tubular vacuolation in the testes. One epididymis or testis was frequently affected more severely relative to the other. These changes were considered unrelated to 2,4-DB administration because they occurred with generally similar incidence and severity relative to control. In addition, these effects were similar to changes caused by thermal stress and immobilization of rats confined in inhalation tubes.

The systemic LOAEC was not determined. The systemic NOAEC is 0.20 mg/L (HDT).

Microscopic changes in the larynx (squamous metaplasia of the laryngeal respiratory and epithelium inflammation of the adjacent connective tissue) were observed at ≥0.02 mg/L after inhalation exposure to 2,4-DB for four weeks. Full recovery of the squamous metaplasia was achieved within four weeks after cessation of exposure to 2,4-DB, with partial recovery achieved for inflammation.